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OXYGEN CONSUMPTION IN ANTERIOR *VERSUS* POSTERIOR EMBRYONIC SHIELD OF *FUNDULUS HETEROCLITUS*

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It has been demonstrated (Brummett, 1968, 1969) that the embryonic shield of *Fundulus* has the fate of its various organs and tissues determined early in gastrulation. When the anterior shield is excised and implanted into the pericardial chamber of an older embryo it differentiates brain and eyes; posterior shield, similarly handled, forms spinal cord, somitic muscle, gut, and other structures appropriate to trunk and tail. Histological studies of these tissues at the time of excision reveal no recognizable differences between the cells of the two shield regions. How, then, might one elucidate and perhaps characterize the differences which the transplantation experiments indicate to be present?

Promising approaches to the above question might include a comparison of the two regions in terms of their fine structure, their biochemistry, and their physiology. The experiments reported here represent an attempt to provide information on one aspect of the third of these approaches.

Differences in respiratory activity of various parts of an egg or developing embryo have long been accepted as indications of differences in extent and direction of differentiation. Barth and Sze (1951) and Sze (1953), for example, measured respiration in various parts of the anuran (*Rana pipiens*) gastrula and established respiratory gradients in that embryo which they related to both cellular interactions (induction) and differentiation.

The objective of the study presented here was to determine whether measurable differences in respiration exist between the anterior and the posterior embryonic shield of the gastrula of the teleost, *Fundulus heteroclitus*.

Materials and Methods

Eggs of *Fundulus heteroclitus* were fertilized in the laboratory. To reduce the possibility of genetic variability in developmental rates, gametes were obtained from one male and one female for each set of experiments. Following fertilization, the eggs were kept at room temperature (approximately 24° C). When the eggs reached the desired stage of gastrulation [Oppenheimer (1937) stages 12, 14 and 16 which correspond to Armstrong and Child (1965) stages 15, 18, and 20], they were washed in 3% formalin-sea water to reduce the possibility of contamination, rinsed in filtered sea water, and dechorionated in sterile Tyrode solution (without bicarbonate) according to methods previously described (Brummett, 1968).

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Operating in sterile Tyrode solution, portions of the embryonic shield were carefully excised with finely sharpened watchmaker's forceps (Brummett, 1968, 1969). Taking care to avoid transferring yolk or periblast along with the tissues, the excised tissue was picked up with a micropipette and placed in a small amount of Tyrode solution in a spotting plate depression. As quickly as possible, each piece was then transferred from the spotting plate to Cartesian diver respirometers in a medium of sterile Tyrode solution. The total volume of the divers ranged from 10–13 μl. All metabolic rate determinations were made at 25° C for a period of approximately two to two and one-half hours.

Operations for weight determinations were performed as above in Tyrode solution. The excised shield parts were rinsed briefly in distilled water to remove most of the balanced salt solution, transferred to a preweighed piece of plastic wrap (Cutrite brand) or aluminum foil, dried overnight at 110° C, and weighed on a microtorsion balance having a sensitivity of ± 2 μg.

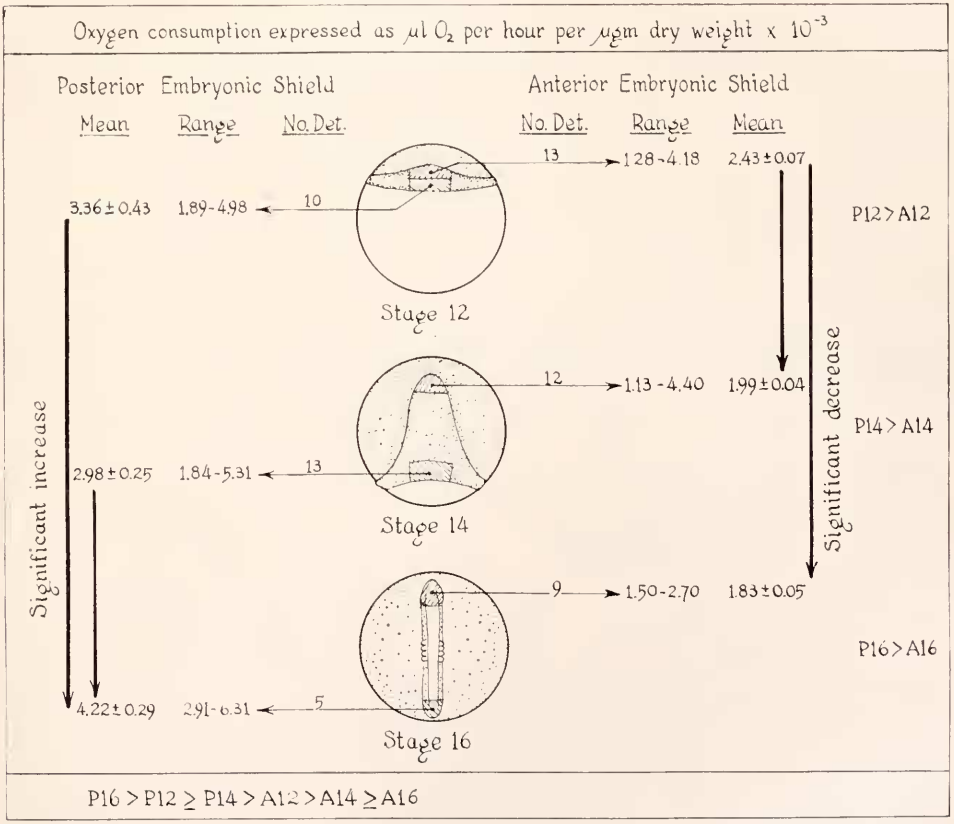

FIGURE 1. Summary of O_2 consumption data for anterior *versus* posterior embryonic shield in three stages of development: early gastrula (stage 12), late gastrula (stage 14), and tail bud stage (16). The number of individual determinations made, the range of measurements obtained, the means ± standard errors for each pool, and significant increases and decreases are indicated.

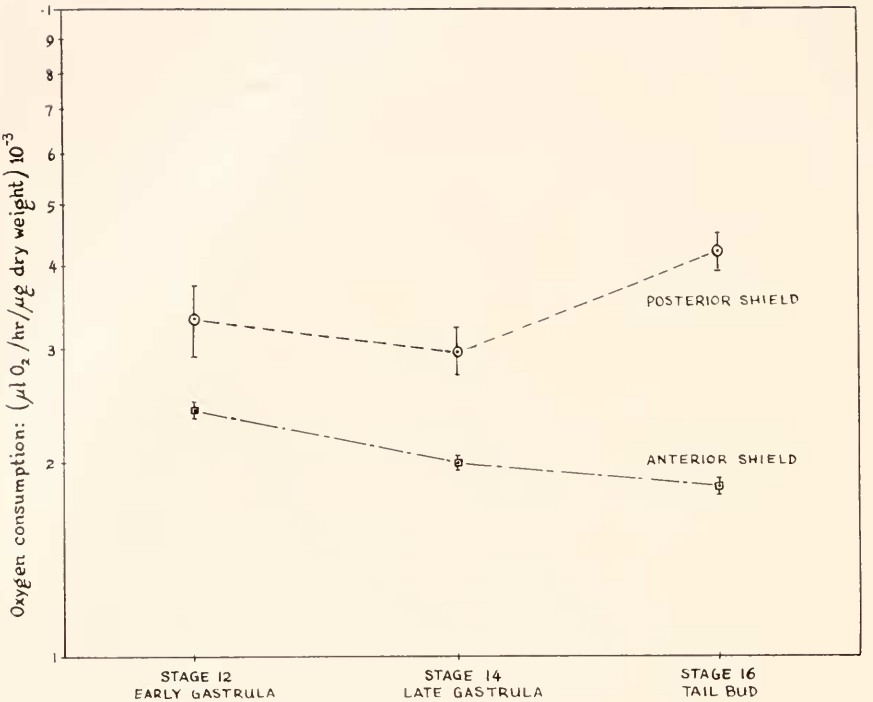

FIGURE 2. Graph comparing O_2 consumption data for anterior (⊡) *versus* posterior (⊙) embryonic shield of early gastrula (stage 12), late gastrula (stage 14), and tail bud stage (16) of *Fundulus heteroclitus* embryos. Standard errors are indicated in each case. Posterior shield measurements are significantly higher than anterior shield measurements at each stage of development and the difference increases as the embryo progresses from early gastrula to tail bud.

Results are expressed as μl O_2/hr per μg dry weight 10^{-3}. Significance of difference of means was determined by the method for small samples given in Simpson, Roe, and Lewontin (1960).

RESULTS

Results are summarized in Figures 1 and 2. The metabolic rate of the posterior shield was significantly greater than that of the anterior shield in all three stages of development studied. Furthermore, these differences tended to increase as gastrulation proceeded: at early gastrula (stage 12) the metabolic rate of the anterior shield was 28% less than that of the posterior shield, at mid-gastrula (stage 14), 33% less, and immediately following blastopore closure (stage 16), 64% less. The level of oxygen uptake in the anterior shield tended to decrease with age; the rate decreasd 18% (significant at $<1\%$ level) between stages 12 and 14 and approximately 8% (not significant) between stages 14 and 16. In contrast, oxygen consumption in the posterior shield decreased 11% (not significant) between stages 12 and 14 but increased approximately 42% between stages 14 and 16 (significant at the $<1\%$ level).

Discussion

Since injured cells will cytolyze, and cytolysis itself will cause changes in O_2 consumption, it is important to point out that explants of the early *Fundulus* embryo can be maintained in Tyrode solution for two days or longer without apparent damage to the cells (Brummett, Haynes, and Pillsbury, unpublished data). The duration of these O_2 consumption experiments was usually less, and never more, than three and one-half hours from the time of excision of the embryonic regions under consideration to termination of the experiment. It seems reasonable to assume then, that the health of the explanted tissues is not a problem in these experiments.

Anterior embryonic shield

The progressive decrease in oxygen consumption in anterior shield fragments as the shield progresses from stage 12 to stage 16 is not consonant with the notion that metabolic rate and hence energy requirement increases as differentiation progresses along a time axis. One is forced to consider other changes in the embryo which might explain the data. One possibility which suggests itself to the authors involves the idea of a progressive decrease in the *movement* of cells in the anterior shield accompanied by a decrease in metabolic rates during this developmental period. Earlier grafting experiments (Brummett, 1969) indicate that at stage 12 the anterior embryonic shield is composed of presumptive forebrain and retinal tissue. Such tissue, excised and implanted into the pericardial chamber of an older embryo, had differentiated brain in 100% and retina in 27% of the sectioned grafts. However, 37% of the *donors* of that tissue developed in an apparently normal fashion and only 13% were totally lacking forebrain and eyes. These results suggest that cells are in the process of migrating into the anterior shield during and perhaps subsequent to early gastrula (stage 12). Such an interpretation is in agreement with the data reported by Ballard and Dodes (1968) on the trout embryo where disengaged cells below the cellular envelope are described as moving into the anterior shield from the central region of the blastodisc during the early stages of epiboly: "As seen from below and confirmed in sagittal sections, the anterior and lateral edges of the gathering embryonic shield, even at six days, still grades off gently and smoothly into the thinned areas of the blastodisc. The convergence movements which bring together the prospective forebrain area of the shield become conspicuous at seven days, whereupon this part of the shield develops sharp boundaries. The outlying thinned area is by then two cells thick, consisting of the cellular envelope and one layer of inner cells" (Ballard and Dodes, 1968, page 77).

Extensive study of developing *Fundulus* embryos suggests that, except for the time factor (the smaller egg of *Fundulus* develops much more rapidly than the trout egg), the quoted description of anterior shield formation in the trout is consonant with the situation in the embryo of *F. heteroclitus*. It seems reasonable to suppose, then, that in early stages of gastrulation there would be considerable movement of cells comprising the early anterior embryonic shield and that, as the shield becomes more definitely formed, the movement would decrease and total oxygen consumption might decrease concomitantly. Significant decrease in cell movement would presumably obscure any increase in metabolic rate associated with progressive differentiation in the stages under consideration here.

Posterior embryonic shield

The increase in oxygen consumption in the posterior embryonic shield during gastrulation is perhaps more difficult to explain. It is almost certain that cells are actively moving into the posterior shield from the lateral shield at both stage 12 and stage 14 (Brummett 1954), and perhaps the amount of activity is essentially the same at these two stages. It seems unlikely, however, that such movement would be *greater* at stage 16 after blastopore closure and the formation of the definitive tail bud. Differentiation has no doubt proceeded further at stage 14 than at stage 12 and still further at stage 16, but in view of the anterior shield picture it would seem presumptuous to attribute the significant increase in oxygen consumption to what would appear to be minor differentiative changes. The question of differential growth comes to mind, but increased mitotic activity in the tail bud blastema must also be set aside as an acceptable explanation of the increase in oxygen consumption in this tissue if one accepts as pertinent Pasteels' (1943) results which revealed essentially no differences in mitotic activity when the blastoporal region was compared with the anterior shield region of the trout embryo. However, this possibility will be considered further below.

Comparison of anterior versus *posterior shield*

The phenomenon of early morphogenesis of brain, common in vertebrate embryos, might lead one to expect earlier and more extensive differentiation in the anterior region of the embryonic shield and hence, perhaps, a higher metabolic rate there than in the posterior shield. Such an expectation is not consistent with the results of these experiments, however, since the posterior shield exhibits a higher rate of oxygen consumption at all three of the stages measured, and the difference increases as gastrulation proceeds. The data suggests, then, that the anterior shield is not significantly ahead of the posterior shield along the differentiative pathway. This interpretation is reasonable in the light of the results of grafting experiments (Brummett, 1968, 1969) which reveal similar time scales for graft differentiation whether the tissue is derived from the anterior or posterior shield. Both donors and grafts of the earlier experiments also demonstrate that even at stage 12, the posterior shield is determined to form trunk and tail structures and the anterior shield is determined to form primarily forebrain, midbrain, and associated structures; presumptive hindbrain is intermediate in position and may be excised with either an anterior or a posterior stage 12 graft. In other words, determined cells appropriate to the entire axis are already present in the early embryonic shield. There is no evidence that presumptive head structures are present first (and hence are differentiatively older), and that presumptive trunk and tail structures enter the shield later as epibody progresses and the shield elongates by virtue of the convergence of cells from the lateral shield and germ ring. With respect to the time axis of differentiation, then, anterior and posterior shield are, in all probability, differentiating simultaneously; *i.e.,* no one area exhibits a precocity of differentiation when compared with other areas of the developing embryo.

The preceding discussion is germane to that aspect of the results which shows that the rate of oxygen consumption in the anterior shield is *not higher* than in the posterior shield. It does not, however, respond to the opposite side of the question:

i.e., why is oxygen consumption in the posterior shield significantly *higher* than in the anterior shield?

It was suspected that differential growth rate might possibly be the source of the observed difference. With this idea in mind the data recorded in the earlier grafting experiments were reviewed, and the time at which obvious growth was first observed and recorded for individual living grafts was noted. When this information for the two series of experiments was compared, it became apparent that in the majority of those cases in which such information was recorded, the posterior grafts exhibited an increase in size *earlier* than did the anterior shield grafts. Indeed, during the first 24 hours following transplantation, growth was noted in 71% (50 of 71 cases) of the posterior grafts as compared with only 8% (3 of 37 cases) of the anterior shield grafts (see Table I). Since the earlier experiments were not designed with this particular question in mind, the records are not as complete as one might wish, but the data agree with the notion that differential growth rate may contribute to higher oxygen consumption in the posterior shield.

TABLE I

Tabulation of growth data (the time when increase in size of graft was first noted) recorded in earlier experiments for anterior and posterior embryonic shield grafts. In general, posterior grafts exhibited increase in size earlier in the post-operative period than did anterior shield grafts

Anterior embryonic shield grafts						Hrs post-operation	Posterior embryonic shield grafts					
	Totals # (%)	St. 12 # (%)	St. 13 # (%)	St. 14 # (%)	St. 15 # (%)		St. 12 # (%)	St. 13 # (%)	St. 14 # (%)	St. 15 # (%)	Totals # (%)	
8%	0 —	0 —	0 —	0 —	0 —	6–12	1 (11)	7 (37)	9 (28)	4 (36)	21 (30)	71%
	3 (8)	0 —	3 (19)	0 —	0 —	18–24	5 (55)	4 (21)	14 (44)	6 (55)	29 (41)	
	2 (5)	1 (8)	1 (6)	0 —	0 —	30–36	2 (22)	2 (11)	6 (19)	0 —	10 (14)	
	10 (27)	2 (15)	4 (25)	1 (20)	3 (100)	42–48	1 (11)	5 (26)	1 (3)	1 (9)	8 (11)	
	6 (16)	2 (15)	3 (19)	1 (20)	0 —	54–60	0 —	0 —	2 (6)	0 —	2 (3)	
	16 (43)	8 (62)	5 (31)	3 (60)	0 —	66–72	0 —	1 (5)	0 —	0 —	1 (1)	
	37	13	16	5	3		9	19	32	11	71	

Mitotic counts should be made to substantiate this interpretation. As was mentioned earlier, however, mitotic counts on the trout embryo by Pasteels (1943) produced negative results.

It is of interest to note that in her studies of metabolic gradients in teleost embryos, Hyman (1921) found that in *Fundulus heteroclitus* the pattern of differential susceptibility to toxins always began at the posterior end of the embryonic axis and proceeded anteriorly. A second susceptibility gradient began subsequently at the anterior end of the axis. Hyman does not provide a great deal of data but what is presented is in agreement with that presented in this paper. Hyman interprets her data to mean that the region of high activity at the posterior end of the embryonic axis reflects a growing point which is responsible for the laying down of the greater part of the *Fundulus* embryo.

Comparison of these results with similar experiments on the amphibian gastrula

Using essentially the same methods described in this paper, Sze (1953) measured oxygen consumption in various portions of the early gastrula of the frog, *Rana*

pipiens. The results of those experiments indicate that oxygen consumption in the frog embryo is less than that in the *Fundulus* embryonic shield by a factor of ten. This is not surprising since the cells of the early amphibian embryo contain a considerable amount of stored yolk which is, presumably, metabolically inert. The cells of the teleost embryo, on the other hand, contain little or no stored yolk but appear to absorb it as needed through the periblast membrane which separates the developing embryo from direct contact with the yolk.

In this study Sze (1953) found that the presumptive neural regions of the early frog gastrula exhibited a higher rate of oxygen consumption than did the presumptive chordamesoderm. Deletion-grafting experiments (Brummett, 1968) demonstrate that the posterior shield tissue in *Fundulus* is not limited to chordamesoderm but consists, rather, of cells which are already determined to form *all* tissues appropriate to posterior trunk and tail. The regions of the frog gastrula measured by Sze (1953), then, are not directly comparable to the teleost tissues used in the experiments reported here.

In another series of experiments in which parts of the frog gastrula in various combinations were subjected to respiration measurements, Barth and Sze (1951) attributed increased oxygen consumption to inductive interactions between "organizer" and presumptive neural plate or presumptive epidermis. Although the teleost posterior embryonic shield has for many years been homologized with the dorsal lip region of the early amphibian embryo, convincing evidence that it is an "organizer" region is lacking. The posterior embryonic shield of the *Fundulus* embryo, however, does contain a wider variety of presumptive tissues than does the anterior shield as revealed by the differentiation of grafts from these two regions (Brummett, 1968, 1969). Nothing is known concerning possible inductive interactions among these various components of the posterior shield in the teleost embryo, but of possible significance is the fact that presumptive notochord appears to be present in the posterior embryonic shield from stage 12 on (Brummett, 1968). In contrast, notochord *never* differentiated in anterior shield grafts of stages 13, 14, and 15, and was found to be present only in those stage 12 grafts which had included more than half of the very early shield (Brummett, 1969). Perhaps, then, the results of the experiments reported here can best be explained in terms of energy-consuming cell interactions (including induction?) which may be significantly greater in the posterior than in the anterior shield at all stages of gastrulation, which may increase in the posterior shield as the embryo goes from stage 12 to stage 16, and which may decrease in the anterior shield during the same period.

It is felt that the experimental data presented in this paper is convincing evidence that measurable physiological differences exist in the posterior *versus* the anterior embryonic shield at a stage when morphological differences in the cells, at the level of magnification possible with light microscopy, is lacking. Electron microscope studies on these tissues are in progress in the hope that they might provide further elucidation of the question of differences. Cell movements, cell proliferation, cell interactions, and cell differentiation are undoubtedly occurring simultaneously in the tissues which were excised and measured in these experiments. These various aspects of cell activity no doubt vary quantitatively in different regions of the embryo and in the same region at different stages of development. They may also vary considerably in their energy requirements. It is difficult, then, to delineate

accurately the causal factors responsible for the differences in oxygen consumption demonstrated by these experiments. We have attempted in the discussion above, however, to consider the possible contribution of each of these important aspects of cell activity to the differences in O_2 uptake obtained in these experiments, and we have tried to interpret the results in the light of what is known about the teleost embryo at this stage of development.

Summary

1. Experiments were designed to determine whether measurable differences in respiration exist between the anterior and posterior embryonic shield of the gastrula of *Fundulus heteroclitus*.

2. Anterior and posterior embryonic shields were carefully excised from embryos of early gastrula, late gastrula, and post-blastopore-closure stages. Using Cartesian diver microrespirometers (10 to 13 μl), oxygen consumption of each individual explant was measured at 25° C over a period of two to two and one-half hours.

3. Oxygen consumption data, expressed as μl O_2 per hour per μg dry weight 10^{-3}, for the two embryonic shield regions at the three stages of development are compared.

4. Posterior shield was found to exhibit a significantly higher rate of O_2 uptake than anterior embryonic shield at all three stages of development.

5. Oxygen uptake appears to increase in the posterior shield as development proceeds from early gastrula to closure of the blastopore; anterior shield exhibits a concomitant decrease.

6. The results are discussed in light of what is known about cell movements, cell proliferation, cell interactions, and cell differentiation in the teleost embryo and are compared with similar experiments on amphibian embryos.

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